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(54) Title: METHOD AND NUCLEIC ACIDS FOR THE ANALYSIS OF ASTROCYTOMAS

(57) Abstract: The present invention relates to chemically modified genomic sequences, to oligonucleotides and/or PNA-oligomers for detecting the cytosine methylation state of genomic DNA, as well as to a method for ascertaining genetic and/or epigenetic parameters of genes for use in the characterisation, classification, differentiation, grading, staging, treatment and/or diagnosis of astrocytomas, or the predisposition to astrocytomas.

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Method and nucleic acids for the analysis of astrocytomas**Field of the Invention**

The levels of observation that have been studied by the methodological developments of recent years in molecular biology, are the genes themselves, the translation of these genes into RNA, and the resulting proteins. The question of which gene is switched on at which point in the course of the development of an individual, and how the activation and inhibition of specific genes in specific cells and tissues are controlled is correlatable to the degree and character of the methylation of the genes or of the genome. In this respect, pathogenic conditions may manifest themselves in a changed methylation pattern of individual genes or of the genome.

The present invention relates to nucleic acids, oligonucleotides, PNA-oligomers, and to a method for the characterisation, classification, differentiation, grading, staging, treatment and/or diagnosis of astrocytomas, or the predisposition to astrocytomas, by analysis of the genetic and/or epigenetic parameters of genomic DNA and, in particular, with the cytosine methylation status thereof.

Prior Art

It is projected that 17,200 adults will develop brain tumors within the United States in 2001. Of the various classes of tumors, gliomas are the most common, of which astrocytomas are one of the most common. These may be graded according to the WHO classification into four categories, pilocytic astrocytomas, low-grade nonpilocytic astrocytomas, anaplastic gliomas, and glioblastomas multiforme. Pilocytic astrocytomas (WHO Grade I) are the most benign, and are usually found in childhood cases. They occasionally form cysts, or are enclosed within cysts, and are slow growing and generally non-invasive. Treatment in the first instance is by surgery, which in some cases may be followed by radiation therapy. The effectiveness of chemotherapy and other forms of treatment are currently being evaluated.

Grade II astrocytomas include fibrillary, gemistocytic and protoplasmic astrocytomas. As opposed to Grade I tumors they are infiltrative. Treatment, is ideally by complete surgical removal, where possible. In some cases surgery may be supplemented by radiation therapy.

A basic property of astrocytic gliomas is an ability to undergo anaplastic change. This is related to the development of serial genetic defects, accounting for the orderly progression of features of malignancy, i.e. hypercellularity, anaplasia. It is important to make the distinction between Grade I pilocytic astrocytomas and diffusely infiltrating Grade II tumors because, it is only the latter group that has a propensity to developing into the malignant Grade III (e.g. anaplastic astrocytoma) and ultimately Grade IV (e.g. glioblastoma multiforme) tumors.

Unlike breast and most other forms of cancer, there are no established guidelines for astrocytoma staging. Diagnosis is most often by scan imaging methods (e.g. MRI, CT) which may be followed by biopsy for histological and cytological analysis. The distinction between Grade I and Grade II astrocytomas may not always be clear using such methods.

Diagnosis by such methodologies does not utilise the molecular basis of the progression to malignancy. Furthermore, molecular markers offer the advantage that even samples of very small sizes and samples whose tissue architecture has not been maintained can be analyzed quite efficiently. Within the last decade numerous genes have been shown to be differentially expressed between benign and malignant tumors. However, no single marker has been shown to be sufficient for the distinction between the two tumors. High-dimensional mRNA based approaches have recently been shown to be able to provide a better means to distinguish between different tumor types and benign and malignant lesions. Application as a routine diagnostic tool in a clinical environment is however impeded by the extreme instability of mRNA, the rapidly occurring expression changes following certain triggers (e.g. sample collection), and, most importantly, the large amount of mRNA needed for analysis (Lipshutz, R. J. et al., *Nature Genetics* 21:20-24, 1999; Bowtell, D. D. L. *Nature genetics suppl.* 21:25-32, 1999), which often cannot be obtained from a routine biopsy.

Aberrant DNA methylation within CpG islands is common in human malignancies leading to abrogation or overexpression of a broad spectrum of genes (Jones, P.A. *Cancer Res* 65:2463-2467, 1996). Abnormal methylation has also been shown to occur in CpG rich regulatory elements in intronic and coding parts of genes for certain tumours (Chan, M.F., et al., *Curr Top Microbiol Immunol* 249:75-86, 2000). Highly characteristic DNA methylation patterns

could also be shown for breast cancer cell lines (Huang, T. H.-M., et al., Hum Mol Genet 8:459-470, 1999).

Abnormal methylation of genes has been linked to the incidence of gliomas (e.g. Epigenetic silencing of PEG3 gene expression in human glioma cell lines. Maegawa *et. al.* Mol Carcinog. 2001 May;31(1):1-9.). It has also been shown that methylation pattern analysis can be correlated with the development of low grade astrocytomas (Aberrant methylation of genes in low-grade astrocytomas. Costello JF, Plass C, Cawene WK. Brain Tumor Pathol. 2000;17(2):49-56). However, the techniques used in such studies (restriction landmark genomic scanning, imprinting analysis) are limited to research, they are unsuitable for use in a clinical or diagnostic setting, and do not provide the basis for the development of a medium or high throughput method for the analysis of gliomas.

5-methylcytosine is the most frequent covalent base modification in the DNA of eukaryotic cells. It plays a role, for example, in the regulation of the transcription, in genetic imprinting, and in tumorigenesis. Therefore, the identification of 5-methylcytosine as a component of genetic information is of considerable interest. However, 5-methylcytosine positions cannot be identified by sequencing since 5-methylcytosine has the same base pairing behavior as cytosine. Moreover, the epigenetic information carried by 5-methylcytosine is completely lost during PCR amplification.

A relatively new and currently the most frequently used method for analyzing DNA for 5-methylcytosine is based upon the specific reaction of bisulfite with cytosine which, upon subsequent alkaline hydrolysis, is converted to uracil which corresponds to thymidine in its base pairing behavior. However, 5-methylcytosine remains unmodified under these conditions. Consequently, the original DNA is converted in such a manner that methylcytosine, which originally could not be distinguished from cytosine by its hybridization behavior, can now be detected as the only remaining cytosine using "normal" molecular biological techniques, for example, by amplification and hybridization or sequencing. All of these techniques are based on base pairing which can now be fully exploited. In terms of sensitivity, the prior art is defined by a method which encloses the DNA to be analyzed in an agarose matrix, thus preventing the diffusion and renaturation of the DNA (bisulfite only reacts with single-stranded DNA), and which replaces all precipitation and purification steps with fast dialysis (Olek A,

- 4 -

Oswald J, Walter J. A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic Acids Res.* 1996 Dec 15;24(24):5064-6). Using this method, it is possible to analyze individual cells, which illustrates the potential of the method. However, currently only individual regions of a length of up to approximately 3000 base pairs are analyzed, a global analysis of cells for thousands of possible methylation events is not possible. However, this method cannot reliably analyze very small fragments from small sample quantities either. These are lost through the matrix in spite of the diffusion protection.

An overview of the further known methods of detecting 5-methylcytosine may be gathered from the following review article: Rein, T., DePamphilis, M. L., Zorbas, H., *Nucleic Acids Res.* 1998, 26, 2255.

To date, barring few exceptions (e.g., Zeschnigk M, Lich C, Buiting K, Doerfler W, Horsthemke B. A single-tube PCR test for the diagnosis of Angelman and Prader-Willi syndrome based on allelic methylation differences at the SNRPN locus. *Eur J Hum Genet.* 1997 Mar-Apr;5(2):94-8) the bisulfite technique is only used in research. Always, however, short, specific fragments of a known gene are amplified subsequent to a bisulfite treatment and either completely sequenced (Olek A, Walter J. The pre-implantation ontogeny of the H19 methylation imprint. *Nat Genet.* 1997 Nov;17(3):275-6) or individual cytosine positions are detected by a primer extension reaction (Gonzalzo ML, Jones PA. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). *Nucleic Acids Res.* 1997 Jun 15;25(12):2529-31, WO 95/00669) or by enzymatic digestion (Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.* 1997 Jun 15;25(12):2532-4). In addition, detection by hybridization has also been described (Olek et al., WO 99/28498).

Further publications dealing with the use of the bisulfite technique for methylation detection in individual genes are: Grigg G, Clark S. Sequencing 5-methylcytosine residues in genomic DNA. *Bioessays.* 1994 Jun;16(6):431-6, 431; Zeschnigk M, Schmitz B, Dittrich B, Buiting K, Horsthemke B, Doerfler W. Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. *Hum Mol Genet.* 1997 Mar;6(3):387-95; Feil R, Charlton J, Bird AP, Walter J, Reik W. Methylation analysis on individual chromosomes: improved protocol for

- 5 -

bisulphite genomic sequencing. Nucleic Acids Res. 1994 Feb 25;22(4):695-6; Martin V, Ribieras S, Song-Wang X, Rio MC, Dante R. Genomic sequencing indicates a correlation between DNA hypomethylation in the 5' region of the pS2 gene and its expression in human breast cancer cell lines. Gene. 1995 May 19;157(1-2):261-4; WO 97/46705, WO 95/15373 and WO 97/45560.

An overview of the Prior Art in oligomer array manufacturing can be gathered from a special edition of Nature Genetics (Nature Genetics Supplement, Volume 21, January 1999), published in January 1999, and from the literature cited therein.

Fluorescently labeled probes are often used for the scanning of immobilized DNA arrays. The simple attachment of Cy3 and Cy5 dyes to the 5'-OH of the specific probe are particularly suitable for fluorescence labels. The detection of the fluorescence of the hybridized probes may be carried out, for example via a confocal microscope. Cy3 and Cy5 dyes, besides many others, are commercially available.

Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-TOF) is a very efficient development for the analysis of biomolecules (Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem. 1988 Oct 15;60(20):2299-301). An analyte is embedded in a light-absorbing matrix. The matrix is evaporated by a short laser pulse thus transporting the analyte molecule into the vapor phase in an unfragmented manner. The analyte is ionized by collisions with matrix molecules. An applied voltage accelerates the ions into a field-free flight tube. Due to their different masses, the ions are accelerated at different rates. Smaller ions reach the detector sooner than bigger ones.

MALDI-TOF spectrometry is excellently suited to the analysis of peptides and proteins. The analysis of nucleic acids is somewhat more difficult (Gut I G, Beck S. DNA and Matrix Assisted Laser Desorption Ionization Mass Spectrometry. Current Innovations and Future Trends. 1995, 1; 147-57). The sensitivity to nucleic acids is approximately 100 times worse than to peptides and decreases disproportionally with increasing fragment size. For nucleic acids having a multiply negatively charged backbone, the ionization process via the matrix is considerably less efficient. In MALDI-TOF spectrometry, the selection of the matrix plays an eminently important role. For the desorption of peptides, several very efficient matrixes have

- 6 -

been found which produce a very fine crystallization. There are now several responsive matrices for DNA, however, the difference in sensitivity has not been reduced. The difference in sensitivity can be reduced by chemically modifying the DNA in such a manner that it becomes more similar to a peptide. Phosphorothioate nucleic acids in which the usual phosphates of the backbone are substituted with thiophosphates can be converted into a charge-neutral DNA using simple alkylation chemistry (Gut IG, Beck S. A procedure for selective DNA alkylation and detection by mass spectrometry. *Nucleic Acids Res.* 1995 Apr 25;23(8):1367-73). The coupling of a charge tag to this modified DNA results in an increase in sensitivity to the same level as that found for peptides. A further advantage of charge tagging is the increased stability of the analysis against impurities which make the detection of unmodified substrates considerably more difficult.

Genomic DNA is obtained from DNA of cell, tissue or other test samples using standard methods. This standard methodology is found in references such as Fritsch and Maniatis eds., *Molecular Cloning: A Laboratory Manual*, 1989.

Description

The disclosed invention provides a method and nucleic acids for the staging of astrocytomas. It discloses a means of distinguishing between healthy tissue, pilocytic astrocytoma (Grade I) and Grade II astrocytoma cells. This provides a means for the improved staging and grading of brain tumors, at a molecular level, as opposed to currently used methods of a relatively subjective nature such as histological analysis and scan imaging. This is of particular importance due to the different prognosis and treatment of Grade I and II astrocytoma patients. The disclosed invention provides the means for the development of a standardised method of astrocytoma staging, which currently does not exist. Furthermore, the disclosed invention presents improvements over the state of the art in that current methods of histological and cytological analysis require that the biopsy contain a sufficient amount of tissue. The method according to the present invention can be used for classification of minute samples.

The invention provides the chemically modified genomic DNA, as well as oligonucleotides and/or PNA-oligomers for detecting cytosine methylations, as well as a method which is particularly suitable for the characterisation, classification, differentiation, grading, staging, treatment and/or diagnosis of astrocytomas. The present invention is based on the discovery

- 7 -

that genetic and epigenetic parameters and, in particular, the cytosine methylation patterns of genomic DNA are particularly suitable for characterisation, classification, differentiation, grading, staging, treatment and/or diagnosis of astrocytomas.

This objective is achieved according to the present invention using a nucleic acid containing a sequence of at least 18 bases in length of the chemically pretreated genomic DNA according to one of Seq. ID No.1 through Seq. ID No.120.

The chemically modified nucleic acid could heretofore not be connected with the ascertainment of disease relevant genetic and epigenetic parameters.

The object of the present invention is further achieved by an oligonucleotide or oligomer for the analysis of chemically pretreated DNA, for detecting the genomic cytosine methylation state, said oligonucleotide containing at least one base sequence having a length of at least 13 nucleotides which hybridizes to a chemically pretreated genomic DNA according to Seq. ID No.1 through Seq. ID No.120. The oligomer probes according to the present invention constitute important and effective tools which, for the first time, make it possible to ascertain specific genetic and epigenetic parameters of brain tumors, in particular, for use in characterisation, classification, differentiation, grading, staging, treatment and/or diagnosis of astrocytomas. The base sequence of the oligomers preferably contains at least one CpG dinucleotide. The probes may also exist in the form of a PNA (peptide nucleic acid) which has particularly preferred pairing properties. Particularly preferred are oligonucleotides according to the present invention in which the cytosine of the CpG dinucleotide is the 5th - 9th nucleotide from the 5'-end of the 13-mer; in the case of PNA-oligomers, it is preferred for the cytosine of the CpG dinucleotide to be the 4th - 6th nucleotide from the 5'-end of the 9-mer.

The oligomers according to the present invention are normally used in so called "sets" which contain at least one oligomer for each of the CpG dinucleotides of the sequences of Seq. ID No.1 through Seq. ID No.120. Preferred is a set which contains at least one oligomer for each of the CpG dinucleotides from one of Seq. ID No.1 through Seq. ID No.120.

Moreover, the present invention makes available a set of at least two oligonucleotides which can be used as so-called "primer oligonucleotides" for amplifying DNA sequences of one of Seq. ID No.1 through Seq. ID No.120, or segments thereof.

In the case of the sets of oligonucleotides according to the present invention, it is preferred that at least one oligonucleotide is bound to a solid phase. It is further preferred that all the oligonucleotides of one set are bound to a solid phase.

The present invention moreover relates to a set of at least 10 n (oligonucleotides and/or PNA-oligomers) used for detecting the cytosine methylation state in chemically pretreated genomic DNA (Seq. ID No.1 through Seq. ID No.120). These probes enable characterisation, classification, differentiation, grading, staging and/or diagnosis of genetic and epigenetic parameters of brain tumors, more specifically astrocytomas. Furthermore, the probes enable the diagnosis of predisposition to astrocytomas. The set of oligomers may also be used for detecting single nucleotide polymorphisms (SNPs) in chemically pretreated genomic DNA according to one of Seq. ID No.1 through Seq. ID No.120.

According to the present invention, it is preferred that an arrangement of different oligonucleotides and/or PNA-oligomers (a so-called "array") made available by the present invention is present in a manner that it is likewise bound to a solid phase. This array of different oligonucleotide- and/or PNA-oligomer sequences can be characterized in that it is arranged on the solid phase in the form of a rectangular or hexagonal lattice. The solid phase surface is preferably composed of silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver, or gold. However, nitrocellulose as well as plastics such as nylon which can exist in the form of pellets or also as resin matrices are possible as well.

Therefore, a further subject matter of the present invention is a method for manufacturing an array fixed to a carrier material for the grading, staging, treatment and/or diagnosis of astrocytomas, in which method at least one oligomer according to the present invention is coupled to a solid phase. Methods for manufacturing such arrays are known, for example, from US Patent 5,744,305 by means of solid-phase chemistry and photolabile protecting groups.

- 9 -

A further subject matter of the present invention relates to a DNA chip for the characterisation, classification, differentiation, grading, staging, treatment and/or diagnosis of astrocytomas. Furthermore the DNA chip enables the diagnosis of predisposition to astrocytomas. The DNA chip contains at least one nucleic acid according to the present invention. DNA chips are known, for example, for US Patent 5,837,832.

Moreover, a subject matter of the present invention is a kit which may be composed, for example, of a bisulfite-containing reagent, a set of primer oligonucleotides containing at least two oligonucleotides whose sequences in each case correspond or are complementary to a 18 base long segment of the base sequences specified in the appendix (Seq. ID No.1 through Seq. ID No.120), oligonucleotides and/or PNA-oligomers as well as instructions for carrying out and evaluating the described method. However, a kit along the lines of the present invention can also contain only part of the aforementioned components.

The present invention also makes available a method for ascertaining genetic and/or epigenetic parameters of genomic DNA. The method is for use in the grading, staging, treatment and/or diagnosis of astrocytomas, in particular for the differentiation of Grade I and Grade II tumors. The method enables the analysis of cytosine methylations and single nucleotide polymorphisms, including the following steps:

In the first step of the method the genomic DNA sample must be isolated from tissue or cellular sources. Such sources may include cell lines, histological slides, body fluids, for example cerebrospinal fluid or lymphatic fluid, or tissue embedded in paraffin; for example, brain, central nervous system or lymphatic tissue. Extraction may be by means that are standard to one skilled in the art, these include the use of detergent lysates, sonification and vortexing with glass beads. Once the nucleic acids have been extracted the genomic double stranded DNA is used in the analysis.

In a preferred embodiment the DNA may be cleaved prior to the chemical treatment, this may be any means standard in the state of the art, in particular with restriction endonucleases.

In the second step of the method, the genomic DNA sample is chemically treated in such a manner that cytosine bases which are unmethylated at the 5'-position are converted to uracil,

- 10 -

thymine, or another base which is dissimilar to cytosine in terms of hybridization behavior. This will be understood as 'chemical pretreatment' hereinafter.

The above described treatment of genomic DNA is preferably carried out with bisulfite (sulfite, disulfite) and subsequent alkaline hydrolysis which results in a conversion of non-methylated cytosine nucleobases to uracil or to another base which is dissimilar to cytosine in terms of base pairing behavior.

Fragments of the chemically pretreated DNA are amplified, using sets of primer oligonucleotides according to the present invention, and a, preferably heat-stable polymerase. Because of statistical and practical considerations, preferably more than ten different fragments having a length of 100 - 2000 base pairs are amplified. The amplification of several DNA segments can be carried out simultaneously in one and the same reaction vessel. Usually, the amplification is carried out by means of a polymerase chain reaction (PCR).

In a preferred embodiment of the method, the set of primer oligonucleotides includes at least two oligonucleotides whose sequences are each reverse complementary or identical to an at least 18 base-pair long segment of the base sequences specified in the appendix (Seq. ID No.1 through Seq. ID No.120). The primer oligonucleotides are preferably characterized in that they do not contain any CpG dinucleotides. In a particularly preferred embodiment of the method, the sequence of said primer oligonucleotides are designed so as to selectively anneal to and amplify, only the astrocytoma and/or brain tissue specific DNA of interest, thereby minimizing the amplification of background or non relevant DNA. In the context of the present invention, background DNA is taken to mean genomic DNA which does not have a relevant tissue specific methylation pattern, in this case the relevant tissue being brain tissue, more specifically astrocyte or astrocytoma tissue. Examples of such primers used in the examples are contained in Table 1.

According to the present invention, it is preferred that at least one primer oligonucleotide is bound to a solid phase during amplification. The different oligonucleotide and/or PNA-oligomer sequences can be arranged on a plane solid phase in the form of a rectangular or hexagonal lattice, the solid phase surface preferably being composed of silicon, glass, poly-

styrene, aluminum, steel, iron, copper, nickel, silver, or gold, it being possible for other materials such as nitrocellulose or plastics to be used as well.

The fragments obtained by means of the amplification can carry a directly or indirectly detectable label. Preferred are labels in the form of fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can be detected in a mass spectrometer, it being preferred that the fragments that are produced have a single positive or negative net charge for better detectability in the mass spectrometer. The detection may be carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

The amplicates obtained in the second step of the method are subsequently hybridized to an array or a set of oligonucleotides and/or PNA probes. In this context, the hybridization takes place in the manner described in the following. The set of probes used during the hybridization is preferably composed of at least 10 oligonucleotides or PNA-oligomers. In the process, the amplicates serve as probes which hybridize to oligonucleotides previously bonded to a solid phase. The non-hybridized fragments are subsequently removed. Said oligonucleotides contain at least one base sequence having a length of 13 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the appendix, the segment containing at least one CpG dinucleotide. The cytosine of the CpG dinucleotide is the 5th to 9th nucleotide from the 5'-end of the 13-mer. One oligonucleotide exists for each CpG dinucleotide. Said PNA-oligomers contain at least one base sequence having a length of 9 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the appendix, the segment containing at least one CpG dinucleotide. The cytosine of the CpG dinucleotide is the 4th to 6th nucleotide seen from the 5'-end of the 9-mer. Preferably one oligonucleotide exists for each CpG dinucleotide.

In the fifth step of the method, the non-hybridized amplicates are removed.

In the final step of the method, the hybridized amplicates are detected. In this context, it is preferred that labels attached to the amplicates are identifiable at each position of the solid phase at which an oligonucleotide sequence is located.

- 12 -

According to the present invention, it is preferred that the labels of the amplicates are fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can be detected in a mass spectrometer. The mass spectrometer is preferred for the detection of the amplicates, fragments of the amplicates or of probes which are complementary to the amplicates, it being possible for the detection to be carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI). The produced fragments may have a single positive or negative net charge for better detectability in the mass spectrometer.

The aforementioned method is preferably used for ascertaining genetic and/or epigenetic parameters of genomic DNA.

The oligomers according to the present invention or arrays thereof as well as a kit according to the present invention are intended to be used for the characterisation, classification, differentiation, grading, staging and/or diagnosis of astrocytomas. More preferably for the differentiation of Grade I and II astrocytomas, or diagnosis of predisposition to astrocytomas. According to the present invention, the method is preferably used for the analysis of important genetic and/or epigenetic parameters within genomic DNA, in particular for use in characterisation, classification, differentiation, grading, staging and/or diagnosis of astrocytomas, and predisposition to astrocytomas.

The method according to the present invention is used, for example, for characterisation, classification, differentiation, grading, staging and/or diagnosis of astrocytomas.

The nucleic acids according to the present invention of Seq. ID No.1 through Seq. ID No.120 can be used for characterisation, classification, differentiation, grading, staging and/or diagnosis of genetic and/or epigenetic parameters of genomic DNA, in particular for use in differentiation of Grade I and II astrocytomas.

The present invention moreover relates to a method for manufacturing a diagnostic reagent and/or therapeutic agent for characterisation, classification, differentiation, grading, staging and/or diagnosis of astrocytomas by analyzing methylation patterns of genomic DNA. The diagnostic reagent and/or therapeutic agent being characterized in that at least one nucleic

acid according to the present invention (sequence IDs 1 through 120) is used for manufacturing it, preferably together with suitable additives and auxiliary agents.

A further subject matter of the present invention relates to a diagnostic reagent and/or therapeutic agent for astrocytoma by analyzing methylation patterns of genomic DNA, in particular for use in differentiation of Grade I and II astrocytomas, or diagnosis of the predisposition to brain tumors, the diagnostic reagent and/or therapeutic agent containing at least one nucleic acid according to the present invention (sequence IDs 1 through 120), preferably together with suitable additives and auxiliary agents.

The present invention moreover relates to the diagnosis and/or prognosis of events which are disadvantageous or relevant to patients or individuals in which important genetic and/or epigenetic parameters within genomic DNA, said parameters obtained by means of the present invention may be compared to another set of genetic and/or epigenetic parameters, the differences serving as the basis for a diagnosis and/or prognosis of events which are disadvantageous or relevant to patients or individuals.

In the context of the present invention the term "hybridization" is to be understood as a bond of an oligonucleotide to a completely complementary sequence along the lines of the Watson-Crick base pairings in the sample DNA, forming a duplex structure.

The term "functional variants" denotes all DNA sequences which are complementary to a DNA sequence, and which hybridize to the reference sequence under stringent conditions.

In the context of the present invention, "genetic parameters" are mutations and polymorphisms of genomic DNA and sequences further required for their regulation. To be designated as mutations are, in particular, insertions, deletions, point mutations, inversions and polymorphisms and, particularly preferred, SNPs (single nucleotide polymorphisms).

In the context of the present invention, "epigenetic parameters" are, in particular, cytosine methylations and further chemical modifications of DNA bases of genomic DNA and sequences further required for their regulation. Further epigenetic parameters include, for exam-

ple, the acetylation of histones which, cannot be directly analyzed using the described method but which, in turn, correlates with the DNA methylation.

In the context of the present invention, the term 'treatment' as applied to astrocytomas is taken to include planning of suitable methods of patient treatment (e.g. surgery, radiation therapy, chemotherapy).

In the following, the present invention will be explained in greater detail on the basis of the sequences and examples with reference to the accompanying figures without being limited thereto.

Figure 1

Figure 1 shows the hybridisation of fluorescent labelled amplicates to a surface bound oligonucleotide. Sample I being from astrocytoma grade I (brain tumor) tissue and sample II being from astrocytoma grade II (brain tumor) tissue. Fluorescence at a spot indicates hybridisation of the amplicate to the oligonucleotide. Hybridisation to a CG oligonucleotide denotes methylation at the cytosine position being analysed, hybridisation to a TG oligonucleotide denotes no methylation at the cytosine position being analysed. It can be seen that Sample I was unmethylated for CG positions (as indicated in example (1-4) of the amplicates of the genes TGF- α (cf. Fig. 1 A), MLH1 (cf. Fig.1 B), NF1 (cf. Fig.1 C) and CSKN2B (Fig1 D) whereas in comparison Sample II had a higher degree of methylation at the same position.

Figure 2

Differentiation of healthy control samples (labelled I) and astrocytoma grade I (labelled II) (Fig.2 A), and healthy control sample and astrocytoma grade II (labelled III) (Fig. 2B). High probability of methylation corresponds to red, uncertainty to black and low probability to green. The labels on the left side of the plot are gene identifiers, the first 3 digits may be referenced in Table 1. The hybridisation was done with Cy5 labelled amplicates generated by multiplex PCR reactions as shown in Table 1. The labels on the right side give the significance (p-value, T-test) of the difference between the means of the two groups. Each row corresponds to a single CpG and each column to the methylation levels of one sample. CpGs are ordered according to their contribution to the distinction to the differential diagnosis of the

two lesions with increasing contribution from top to bottom.

Figure 3

Differentiation of astrocytoma grade I (1) and astrocytoma grade II (2). High probability of methylation corresponds to red, uncertainty to black and low probability to green. The labels on the left side of the plot are gene and CpG identifiers. The hybridisation was done with Cy5 labelled amplicates generated by multiplex PCR reactions as shown in Table 1. The labels on the right side give the significance (p-value, T-test) of the difference between the means of the two groups. Each row corresponds to a single CpG and each column to the methylation levels of one sample. CpGs are ordered according to their contribution to the distinction to the differential diagnosis of the two lesions with increasing contribution from top to bottom.

Figure 4

Separation of astrocytoma grade I (I) and astrocytoma grade II (II). High probability of methylation corresponds to red, uncertainty to black and low probability to green. The labels on the left side of the plot are gene and CpG identifiers. The hybridisation was done with Cy5 labelled amplicates of the genes MLHI, TGF-alpha and NF1, all generated by single gene PCR reactions. Each row corresponds to a single CpG and each column to the methylation levels of one sample. CpGs are ordered according to their contribution to the distinction to the differential diagnosis of the two lesions with increasing contribution from top to bottom.

Seq. ID No. 1 through Seq. ID No. 120

Sequences having odd sequence numbers (e.g., Seq. ID No. 1, 3, 5, ...) exhibit in each case sequences of chemically pretreated genomic DNAs. Sequences having even sequence numbers (e.g., Seq. ID No. 2, 4, 6, ...) exhibit in each case the sequences of chemically pretreated genomic DNAs. Said genomic DNAs are complementary to the genomic DNAs from which the preceding sequence was derived (e.g., the complementary sequence to the genomic DNA from which Seq. ID No.1 is derived is the genomic sequence from which Seq. ID No.2 is derived, the complementary sequence to the genomic DNA from which Seq. ID No.3 is derived is the sequence from which Seq. ID No.4 is derived, etc.)

Seq. ID No. 121 through Seq. ID No. 136

Seq. ID No. 121 through Seq. ID No. 136 show the sequences of oligonucleotides that are used in the following Examples.

Example 1 :Methylation analysis of the gene TGF-alpha.

The following example relates to a fragment of the gene TGF-alpha in which a specific CG-position is to be analyzed for methylation.

In the first step, a genomic sequence is treated using bisulfite (hydrogen sulfite, disulfite) in such a manner that all cytosines which are not methylated at the 5-position of the base are modified in such a manner that a different base is substituted with regard to the base pairing behavior while the cytosines methylated at the 5-position remain unchanged.

If bisulfite solution is used for the reaction, then an addition takes place at the non-methylated cytosine bases. Moreover, a denaturing reagent or solvent as well as a radical interceptor must be present. A subsequent alkaline hydrolysis then gives rise to the conversion of non-methylated cytosine nucleobases to uracil. The chemically converted DNA is then used for the detection of methylated cytosines. In the second method step, the treated DNA sample is diluted with water or an aqueous solution. Preferably, the DNA is subsequently desulfonated. In the third step of the method, the DNA sample is amplified in a polymerase chain reaction, preferably using a heat-resistant DNA polymerase. In the present case, cytosines of the gene TGF-alpha are analyzed. To this end, a defined fragment having a length of 533bp is amplified with the specific primer oligonucleotides GGTTTGTTTGGGAGGTAAG (Sequence ID 121) and CCCCTAAAAACACAAAA (Sequence ID No. 122). The single gene PCR reaction was performed on a thermocycler (Eppendorf GmbH) using bisulfite DNA 10 ng, primer 6 pmole each, dNTP 200 µM each, 1.5 mM MgCl₂ and 1 U HotstartTaq (Qiagen AG). The other conditions were as recommended by the Taq polymerase manufacturer. In the multiplex PCR up to 16 primer pairs were used within the PCR reaction. The multiplex PCR was done according the single gene PCR with the following modifications: primer 0.35 pmole each, dNTP 800 µM each and 4,5 mM MgCl₂. The cycle program for single gene PCR and multiplex PCR was as followed: step 1, 14 min 96 °C; step 2, 60 sec 96°C; step 3, 45 sec 55 °C; step 4, 75 sec 72 °C; step 5, 10 min 72 °C; the step 2 to step 4 were repeated 39 fold.

- 17 -

The amplificate serves as a sample which hybridizes to an oligonucleotide previously bound to a solid phase, forming a duplex structure, for example AAGTTAGGCGTTTTTTGT (Sequence ID No. 123), the cytosine to be detected being located at position 382 of the amplificate. The detection of the hybridization product is based on Cy3 and Cy5 fluorescently labelled primer oligonucleotides which have been used for the amplification. A hybridization reaction of the amplified DNA with the oligonucleotide takes place only if a methylated cytosine was present at this location in the bisulfite-treated DNA. Thus, the methylation status of the specific cytosine to be analyzed is inferred from the hybridization product.

In order to verify the methylation status of the position, a sample of the amplificate is further hybridized to another oligonucleotide previously bonded to a solid phase. Said oligonucleotide is identical to the oligonucleotide previously used to analyze the methylation status of the sample, with the exception of the position in question. At the position to be analysed said oligonucleotide comprises a thymine base as opposed to a cytosine base i.e. AAGTTAGGTGTTTTTTGT (Sequence ID No. 124). Therefore, the hybridisation reaction only takes place if an unmethylated cytosine was present at the position to be analysed.

Example 2: Methylation analysis of the gene NF1.

The following example relates to a fragment of the gene NF1 in which a specific CG-position is to be analyzed for methylation.

In the first step, a genomic sequence is treated using bisulfite (hydrogen sulfite, disulfite) in such a manner that all cytosines which are not methylated at the 5-position of the base are modified in such a manner that a different base is substituted with regard to the base pairing behavior while the cytosines methylated at the 5-position remain unchanged.

If bisulfite solution is used for the reaction, then an addition takes place at the non-methylated cytosine bases. Moreover, a denaturing reagent or solvent as well as a radical interceptor must be present. A subsequent alkaline hydrolysis then gives rise to the conversion of non-methylated cytosine nucleobases to uracil. The chemically converted DNA is then used for the detection of methylated cytosines. In the second method step, the treated DNA sample is diluted with water or an aqueous solution. Preferably, the DNA is subsequently desulfonated. In the third step of the method, the DNA sample is amplified in a polymerase chain reaction,

preferably using a heat-resistant DNA polymerase. In the present case, cytosines of the gene NF1 are analyzed. To this end, a defined fragment having a length of 600 bp is amplified with the specific primer oligonucleotides TTGGGAGAAAGGTTAGTTTT (Sequence ID 129) and ATACAAACTCCCAATATTCC (Sequence ID No. 130). The single gene PCR reaction was performed on a thermocycler (Eppendorf GmbH) using bisulfite DNA 10 ng, primer 6 pmole each, dNTP 200 μ M each, 1.5 mM MgCl₂ and 1 U HotstartTaq (Qiagen AG). The other conditions were as recommended by the Taq polymerase manufacturer. In the multiplex PCR up to 16 primer pairs were used within the PCR reaction. The multiplex PCR was done according the single gene PCR with the following modifications: primer 0.35 pmole each, dNTP 800 μ M each and 4,5 mM MgCl₂. The cycle program for single gene PCR and multiplex PCR was as followed: step 1, 14 min 96 °C; step 2, 60 sec 96°C; step 3, 45 sec 55 °C; step 4, 75 sec 72 °C; step 5, 10 min 72 °C; the step 2 to step 4 were repeated 39 fold.

The amplificate serves as a sample which hybridizes to an oligonucleotide previously bound to a solid phase, forming a duplex structure, for example AATTAAAACGCCCTAAAA (Sequence ID No. 131), the cytosine to be detected being located at position 24 of the amplificate. The detection of the hybridization product is based on Cy3 and Cy5 fluorescently labelled primer oligonucleotides which have been used for the amplification. A hybridization reaction of the amplified DNA with the oligonucleotide takes place only if a methylated cytosine was present at this location in the bisulfite-treated DNA. Thus, the methylation status of the specific cytosine to be analyzed is inferred from the hybridization product.

In order to verify the methylation status of the position, a sample of the amplificate is further hybridized to another oligonucleotide previously bonded to a solid phase. Said oligonucleotide is identical to the oligonucleotide previously used to analyze the methylation status of the sample, with the exception of the position in question. At the position to be analysed said oligonucleotide comprises a thymine base as opposed to a cytosine base i.e. AATTAAAACACCCTAAAA (Sequence ID No. 132). Therefore, the hybridisation reaction only takes place if an unmethylated cytosine was present at the position to be analysed.

Example 3: Methylation analysis of the gene MLH1.

The following example relates to a fragment of the gene MLH1 in which a specific CG-position is to be analyzed for methylation.

In the first step, a genomic sequence is treated using bisulfite (hydrogen sulfite, disulfite) in such a manner that all cytosines which are not methylated at the 5-position of the base are modified in such a manner that a different base is substituted with regard to the base pairing behavior while the cytosines methylated at the 5-position remain unchanged.

If bisulfite solution is used for the reaction, then an addition takes place at the non-methylated cytosine bases. Moreover, a denaturing reagent or solvent as well as a radical interceptor must be present. A subsequent alkaline hydrolysis then gives rise to the conversion of non-methylated cytosine nucleobases to uracil. The chemically converted DNA is then used for the detection of methylated cytosines. In the second method step, the treated DNA sample is diluted with water or an aqueous solution. Preferably, the DNA is subsequently desulfonated. In the third step of the method, the DNA sample is amplified in a polymerase chain reaction, preferably using a heat-resistant DNA polymerase. In the present case, cytosines of the gene MLHI are analyzed. To this end, a defined fragment having a length of 568 bp is amplified with the specific primer oligonucleotides TTTAAGGTAAGAGAATAGGT (Sequence ID 133) and TTAACCCTACTCTTATAACC (Sequence ID No. 134). The single gene PCR reaction was performed on a thermocycler (Eppendorf GmbH) using bisulfite DNA 10 ng, primer 6 pmole each, dNTP 200 μ M each, 1.5 mM MgCl₂ and 1 U HotstartTaq (Qiagen AG). The other conditions were as recommended by the Taq polymerase manufacturer. In the multiplex PCR up to 16 primer pairs were used within the PCR reaction. The multiplex PCR was done according the single gene PCR with the following modifications: primer 0.35 pmole each, dNTP 800 μ M each and 4,5 mM MgCl₂. The cycle program for single gene PCR and multiplex PCR was as followed: step 1, 14 min 96 °C; step 2, 60 sec 96°C; step 3, 45 sec 55 °C; step 4, 75 sec 72 °C; step 5, 10 min 72 °C; the step 2 to step 4 were repeated 39 fold.

The amplificate serves as a sample which hybridizes to an oligonucleotide previously bound to a solid phase, forming a duplex structure, for example TTGTAGGACGTTTATATG (Sequence ID No. 135), the cytosine to be detected being located at position 125 of the amplificate. The detection of the hybridization product is based on Cy3 and Cy5 fluorescently labelled primer oligonucleotides which have been used for the amplification. A hybridization reaction of the amplified DNA with the oligonucleotide takes place only if a methylated cyto-

- 20 -

sine was present at this location in the bisulfite-treated DNA. Thus, the methylation status of the specific cytosine to be analyzed is inferred from the hybridization product.

In order to verify the methylation status of the position, a sample of the amplificate is further hybridized to another oligonucleotide previously bonded to a solid phase. Said oligonucleotide is identical to the oligonucleotide previously used to analyze the methylation status of the sample, with the exception of the position in question. At the position to be analysed said oligonucleotide comprises a thymine base as opposed to a cytosine base i.e TTGTAGGATGTTTATATG (Sequence ID No. 136). Therefore, the hybridisation reaction only takes place if an unmethylated cytosine was present at the position to be analysed.

Example 4: Methylation analysis of the gene CSNK2B.

The following example relates to a fragment of the gene CSNK2B in which a specific CG-position is to be analyzed for methylation.

In the first step, a genomic sequence is treated using bisulfite (hydrogen sulfite, disulfite) in such a manner that all cytosines which are not methylated at the 5-position of the base are modified in such a manner that a different base is substituted with regard to the base pairing behavior while the cytosines methylated at the 5-position remain unchanged.

If bisulfite solution is used for the reaction, then an addition takes place at the non-methylated cytosine bases. Moreover, a denaturing reagent or solvent as well as a radical interceptor must be present. A subsequent alkaline hydrolysis then gives rise to the conversion of non-methylated cytosine nucleobases to uracil. The chemically converted DNA is then used for the detection of methylated cytosines. In the second method step, the treated DNA sample is diluted with water or an aqueous solution. Preferably, the DNA is subsequently desulfonated. In the third step of the method, the DNA sample is amplified in a polymerase chain reaction, preferably using a heat-resistant DNA polymerase. In the present case, cytosines of the gene CSNK2B are analyzed. To this end, a defined fragment having a length of 524 bp is amplified with the specific primer oligonucleotides GGGGAAATGGAGAAGTGTA (Sequence ID 125) and CTACCAATCCCAAATAACC (Sequence ID No. 126). The single gene PCR reaction was performed on a thermocycler (Eppendorf GmbH) using bisulfite DNA 10 ng, primer 6 pmole each, dNTP 200 μ M each, 1.5 mM MgCl₂ and 1 U HotstartTaq (Qiagen AG).

- 21 -

The other conditions were as recommended by the Taq polymerase manufacturer. In the multiplex PCR up to 16 primer pairs were used within the PCR reaction. The multiplex PCR was done according the single gene PCR with the following modifications: primer 0.35 pmole each, dNTP 800 μ M each and 4,5 mM MgCl₂. The cycle program for single gene PCR and multiplex PCR was as followed: step 1, 14 min 96 °C; step 2, 60 sec 96°C; step 3, 45 sec 55 °C; step 4, 75 sec 72 °C; step 5, 10 min 72 °C; the step 2 to step 4 were repeated 39 fold.

The amplificate serves as a sample which hybridizes to an oligonucleotide previously bound to a solid phase, forming a duplex structure, for example TAGGTTAGCGTATTGGGA (Sequence ID No. 127), the cytosine to be detected being located at position 50 of the amplificate. The detection of the hybridization product is based on Cy3 and Cy5 fluorescently labelled primer oligonucleotides which have been used for the amplification. A hybridization reaction of the amplified DNA with the oligonucleotide takes place only if a methylated cytosine was present at this location in the bisulfite-treated DNA. Thus, the methylation status of the specific cytosine to be analyzed is inferred from the hybridization product.

In order to verify the methylation status of the position, a sample of the amplificate is further hybridized to another oligonucleotide previously bonded to a solid phase. Said oligonucleotide is identical to the oligonucleotide previously used to analyze the methylation status of the sample, with the exception of the position in question. At the position to be analysed said oligonucleotide comprises a thymine base as opposed to a cytosine base i.e. TAGGTTAGTGTATTGGGA (Sequence ID No. 128). Therefore, the hybridisation reaction only takes place if an unmethylated cytosine was present at the position to be analysed.

Example 5: Differentiation of healthy samples and astrocytoma grade I and grade II tumours isolated from cerebrum

In order to relate the methylation patterns to a specific tumour type, it is initially required to comparatively analyze the DNA methylation patterns of two groups of patients with alternative forms of a tumor, in this case one group of astrocytoma grade I and another group of astrocytoma grade II, with those of healthy tissue (Fig 2 A and B). These analyses were carried out, analogously to Examples 1-4. The results obtained in this manner are stored in a database and the CpG dinucleotides which are methylated differently between the two groups are identified. This can be carried out by determining individual CpG methylation rates as can be

- 22 -

done, for example, by sequencing, which is a relatively imprecise method of quantifying methylation at a specific CpG, or else, in a very precise manner, by a methylation-sensitive "primer extension reaction". In a particularly preferred variant, as illustrated in the preceeding examples the methylation status of hundreds or thousands of CpGs may be analysed on an oligomer array. It is also possible for the patterns to be compared, for example, by clustering analyses which can be carried out, for example, by a computer.

All clinical specimens were obtained at time of surgery, i.e. in a routine clinical situation (Santourlidis, S., Prostate 39:166-174, 1999, Florl, A.R., Br. J. Cancer 80:1312-1321, 1999). A panel of genomic fragments from 64 different genes (listed in Table 1) were bisulphite treated and amplified by 6 sets of multiplex PCRs (mPCR) according to Example 1. The mPCR reactions (I,J,K,L,M,N) of the genomic, bisulphite treated DNA was done using the combination of primer pairs as indicated in Table 1. However, as will be obvious to one skilled in the art, it is also possible to use other primers that amplify the genomic, bisulphite treated DNA in an adequate manner. However the primer pairs as listed in Table 1 are particularly preferred. In order to differentiate astrocytoma grade I from healthy control samples optimal results were obtained by including at least 6 CpG dinucleotides, the most informative CpG positions for this discrimination being located within the OAT, GP1B, cMyc, UNG, TIMP3 and cABL genes (cf. Fig. 2A, Tab1). In order to differentiate astrocytoma grade I from healthy control samples optimal results were obtained by including at least 6 CpG dinucleotides, the most informative CpG positions for this discrimination being located within the cMyc, EGR4, ApoA1, AR and heatshock genes (cf. Fig. 2B, Tab1). In addition, the majority of the analysed CpG dinucleotides of the panel showed different methylation patterns between the two phenotypes. The results prove that methylation fingerprints are capable of providing differential diagnosis of solid malignant tumours and could therefore be applied in a large number clinical situations

Example 6: Differentiation of astrocytoma grade I and grade II tumours

In order to relate the methylation patterns to a specific tumour type, it is initially required to analyze the DNA methylation patterns of two groups of patients with alternative forms of a tumor, in this case one group of astrocytoma grade I and another group of astrocytoma grade II. These analyses were carried out, analogously to Example 1. The results obtained in this manner are stored in a database and the CpG dinucleotides which are methylated differently

- 23 -

between the two groups are identified. This can be carried out by determining individual CpG methylation rates as can be done, for example, by sequencing, which is a relatively imprecise method of quantifying methylation at a specific CpG, or else, in a very precise manner, by a methylation-sensitive "primer extension reaction". In a particularly preferred variant, as illustrated in examples 1 to 4 the methylation status of hundreds or thousands of CpGs may be analysed on an oligomer array. It is also possible for the patterns to be compared, for example, by clustering analyses which can be carried out, for example, by a computer.

All clinical specimens were obtained at time of surgery, i.e. in a routine clinical situation (Santourlidis, S., Prostate 39:166-174, 1999, Florl, A.R., Br. J. Cancer 80:1312-1321, 1999). A panel of genomic fragments from 56 different genes (listed in Table 1) were bisulphite treated and amplified by 6 sets of multiplex PCRs (mPCR), named I,J,K,L,M and N, in Table 1, according to Example 1. The mPCR reactions of the genomic, bisulphite treated DNA was done using the combination of primer pairs as indicated in Table 1. It will be obvious to one skilled in the art, that it is also possible to use other primers that amplify the genomic, bisulphite treated DNA in an adequate manner. However the primer pairs as listed in Table 1 are particularly preferred. Optimal results were obtained by including at least 8 CpG dinucleotides, the most informative CpG positions for this discrimination being located within the CSKNB2, NF1, MIH1, EGR4, AR; TGF-alpha, and APOC2 genes (cf. Fig. 3). In addition, the majority of the analysed CpG dinucleotides of the panel showed different methylation patterns between the two phenotypes. The results prove that methylation fingerprints are capable of providing differential diagnosis of solid malignant tumours and could therefore be applied in a large number clinical situations.

Example 7: Differentiation of astrocytoma grade I and grade II tumours using DNA fragments derived from TGF-alpha, NF1 and MIH1 gene.

The methylation patterns of CpG islands derived from TGF-alpha, NF1 and MIH1 genes were analysed. In order to evaluate the genes, already identified differentiating astrocytoma grade I and grade II tumours in the class prediction approach (cf. Example 6) The genes TGF-alpha, NF1 and MIH1 gene were amplified from genomic bisulfite treated DNA as described in examples 1,2 and 3. The DNA was prepared from tissue samples of two groups of patients with alternative forms of a tumor, in this case one group of astrocytoma grade I and another group of astrocytoma grade II. Optimal results were obtained by including at least 6 CpG dinucleo-

tides, the most informative CpG positions for this discrimination being located within the TGF- α and NF1 and M1H1 genes (cf. Fig. 4). The results further validate the results of methylation fingerprints shown in example 6.

Table 1

List of genes, reference numbers and primer oligonucleotides according to Examples 1-7 and figures 1-4.

GENE ID	MPCR SET	GENE	PCR PRIMER	PCR PRIMER
81	N	ADCYAP1	GGTGGATTATGGTTA TTTTG	TCCCTCCCTTACCCTTCAAC
292	K	AFP	AGGTTTATTGAATATTT AGG	AACATATTTCCACAACATCC
85	L	ANT1	GTTTAAGGTTGTTTGTG TTATAAAT	CCTCCTCCCAACTACAAAA
48	L	APOA1	GTTGGTGGTGGGGGAG GTAG	ACAACCAAAATCTAAACTAA
50	N	APOC2	ATGAGTAGAAGAGGTG ATAT	CCCTAAATCCCTTTCTTACC
87	K	AR	GTAGTAGTAGTAGTAA GAGA	ACCCCTAAATAATTATCCT
1143	L	ATP5A1	AGTTTGTTTAAATTTAT TGATAGGA	AACAACATCTTACAATTACTCC
1011	L	CABL	GGTTGGGAGATTTAAT TTTATT	ACCAATCCAAACTTTTCCTT
77	L	CD1A	ATTATGGTTGGAATTG TAAT	ACAAAAACAACAAACACCCC
1079	L	CD63	TGGGAGATATTTAGGA TGTGA	CTCACCTAAACTTCCCAA
99	M	CDC25A	AGAAGTTGTTTATTGA TTGG	AAAATTAAATCCAAACAAAC
187	L	CDH3	GTTTAGAAGTTTAAGA TTAG	CAAAACTCAACCTCTATCT
88	K	CDK4	TTTTGGTAGTTGGTTAT ATG	AAAAATAACACAATAACTCA
310	I	CFOS	TTTTGAGTTTLAGAATT GTTTTTAG	AAAAACCCCTACTCATCTACTA
1034	L	CMYC	TTTTGTGTGGAGGGTA GTTG	CCCCAAATAAACAAAATAACC
312	K	CMYC	TTGTTTTTGTGGAAAA GAGG	TTTCAATCTCAAACTCAACC
313	I	CMYC	AAAGGTTTGGAGGTAG GAGT	TTCTTTCCAAATCCTCTTT
37	M	CRIP1	TTTAGGTTTAGGGTTTA GTT	CCACTCCAAAATAATATCA
70	N	CSF1	TAGGGTTTGGAGGGAA AG	AAAAATCACCCCTAACCAAAAC
78	M	CSNK2B	GGGGAAATGGAGAAG TGTA	CTACCAATCCCAAAATAACC
272	N	CTLA4	TTTTTATGGAGAGTAG TTGG	TAACTTTACTCACCAATTAC
287	K	DAD1	TTTTGTGTAGAGTAA TTG	ACCTCAATTTCCCCATTAC
147	I	DAPK1	ATTAATATTATGTAAA GTGA	CTTACAACCATTCACCCACA

GENE ID	MPCR SET	GENE	PCR PRIMER	PCR PRIMER
319	J	E-CADHERIN	GGGTGAAAGAGTGAGT TTTATTT	ACTCCAAAAACCCATAACTAA
63	M	EGFR	GGTGTGATAAGATT TGAAG	CCCTTACCTTCTTTTCCT
311	I	EGFR	GGGTAGTGGGATATTT AGTTTT	CCAACACTACCCCTCTAA
82	M	EGR4	AGGGGGATTGAGTGTT AAGT	CCCAAACATAAACACAAAAT
1012	L	ELK1	AAGTGTGTTAGTTTTTA ATGGGTA	CAAACCCAAAACCTCACCTAT
307	J	ERBB2	GAGTGATATTTTATTT TATGTTGG	AAAACCCTAACTCAACTACTCAC
308	K	ERBB2	GAGTTTGGGAGTTTAA GATTAGT	TCAACTTCACAACCTTCATTCTTAT
130	N	GP1B	GGTGATAGGAGAATAA TGTTGG	TCTCCCAACTACAACCAAAC
290.2	M	HEAT SHOCK	AGAGGAGATATTTTT ATGG	AAAAATCCTACAACAACCTTC
290.3	J	HEAT SHOCK	AAGGATAATAATTTGT TGGG	CTTAAATACAACTTAATCC
89	I	HUMOS	TTTATTGATTGGGAGT AGGT	CTAATTTACAAACATCCTA
1083	N	IL13	TTTTTAGGGTAGGGGT TGT	CCTTATCCCCCATAACCA
1010	L	LMYC	AGGTTTGGGTATTGA GTTT	CATTATTTCTAACTACCTTATAT CTC
291	L	MC2R	ATATTTGATATGTTGG GTAG	ACCTACTACAAAAATCATC
314.2	I	MGMT	AAGGTTTtagggaaga GTGTTT	ACTCCCAATACCTCACAATATAA C
427	K	MHC	GGGTATTAGGAATTTA TGTTG	CAAAACACCTTCCTAACTCA
401	I	MHC	TTGTTGTTTTAGGGGT TTTGG	TCCTTCCCATTCTCCAAATATC
458	M	MHC	AAGAGTGAGAAGTAG AGGGTT	CTACTCTCTAAAACCTCCAAAC
487	M	MHC	GAGGTAAAGGAAGTT TTGGA	AAACTAAATTCTCCCAATACC
465	L	MHC	ATTGATAGGTAGTTAG ATTGG	AAAAAACTCTCATAAATCTCA
451	M	MHC	AGGAGGAAGGGTTAAT AAAGA	ATCTTCCTACTACTATCTCTAAC
441	M	MHC	AGGTTGGATTTTGGGT AGGT	TCTCCTACTCTCCTAATCTC
160	M	MLH1	TTTAAGGTAAGAGAAT AGGT	TTAACCTACTCTTATAACC
94	N	N33	TGGAGGAGATATTGTT TTGT	TTTTTCAAATCAAACCCCTACT
302	J	NF1	TTGGGAGAAAGGTTAG TTTT	ATACAAACTCCCAATATTCC
1009	L	NMYC	GGAGGAGTATATTTTG GGTTT	ACAAACCCTACTCCTTACCTC
1018	N	NUC	AAGTTGTGTTTTTAAA AGGGTTA	AAAAACTAAACCTACCCAATAA
1007	N	OAT	TGGAGGTGGATTTAGA GGTA	ACCAAACCCCAAAACAA
304	J	P16	AGGGGTTGGTTGGTTA TTAG	TAATCCAATTCCCCTACAA

- 26 -

GENE ID	MPCR SET	GENE	PCR PRIMER	PCR PRIMER
305	J	P53	GTGATAAGGGTTGTGA AGGA	CAAAAACCTTACCCAATCCAA
1069	N	POMC	AGTTTTTAAATAATGG GGAAAT	ACTCTTCTTCCCCTCCTTC
177	N	PRG	AGTTGAAGTTATAAGG GGTG	AATAAAAACTCTCAAAAACC
26	K	SOD1	AGGGGAAGAAAAGGT AAGTT	CCCACTCTAACCCCAAACCA
303	I	TGF-A	GGTTTGTGTTGGGAGGT AAG	CCCCCTAAAAACACAAAA
301	J	TGF-B1	GGGGAGTAATATGGAT TTGG	CCTTTACTAAACACCTCCCATA
317	I	TIMP3	GTAAGGGTTTTGTGTT GTTT	CCCCCTCAAACCAATAAC
128	N	TNFB	TTTTTGTTTTTGATTGA AATAGTAG	AAAAACCCCAAATAAACAA
35	L	UBB	TTAAGTTATTTTAGGTG GAGTTTA	ACCAAATCCTACCAATCAC
1140	N	UNG	GTTGGGGTGTTTGAGG AA	CCTCTCCCCTCTAATTAAACA
300	J	VEGF	TGGGTAATTTTAGGTT GTGA	CCCCAAAAACAAATCACTC
188	K	WT1	AAAGGGAAATTAAGTG TTGT	TAACTACCCTCAACTTCCC

- 27 -

Claims

1. A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated genomic DNA according to one of the sequences taken from the group of Seq. ID No.1 to Seq. ID No.120 and sequences complementary thereto.
2. An oligomer, in particular an oligonucleotide or peptide nucleic acid (PNA)-oligomer, said oligomer comprising in each case at least one base sequence having a length of at least 9 nucleotides which hybridizes to or is identical to a chemically pretreated genomic DNA according to one of the Seq ID Nos 1 to 120 according to claim 1.
3. The oligomer as recited in Claim 2; wherein the base sequence includes at least one CpG dinucleotide.
4. The oligomer as recited in Claim 2; characterized in that the cytosine of the CpG dinucleotide is located approximately in the middle third of the oligomer.
5. A set of oligomers, comprising at least two oligomers according to any of claims 2 to 4.
6. A set of oligomers as recited in Claim 5, comprising oligomers for detecting the methylation state of all CpG dinucleotides within one of the sequences according to Seq. ID Nos. 1 through 120 according to claim 1, and sequences complementary thereto.
7. A set of at least two oligonucleotides as recited in Claim 2, which can be used as primer oligonucleotides for the amplification of DNA sequences of one of Seq. ID 1 through Seq. ID 120 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto and segments thereof.
8. A set of oligonucleotides as recited in Claim 7, characterized in that at least one oligonucleotide is bound to a solid phase.

- 28 -

9. Use of a set of oligomer probes comprising at least ten of the oligomers according to any of claims 5 through 8 for detecting the cytosine methylation state and/or single nucleotide polymorphisms (SNPs) in a chemically pretreated genomic DNA according to claim 1.
10. A method for manufacturing an arrangement of different oligomers (array) fixed to a carrier material for analyzing diseases associated with the methylation state of the CpG dinucleotides of one of the Seq. ID 1 through Seq. ID 120 and sequences complementary thereto, wherein at least one oligomer according to any of the claims 2 through 4 is coupled to a solid phase.
11. An arrangement of different oligomers (array) obtainable according to claim 10.
12. An array of different oligonucleotide- and/or PNA-oligomer sequences as recited in Claim 11, characterized in that these are arranged on a plane solid phase in the form of a rectangular or hexagonal lattice.
13. The array as recited in any of the Claims 11 or 12, characterized in that the solid phase surface is composed of silicon, glass, polystyrene, aluminium, steel, iron, copper, nickel, silver, or gold.
14. A DNA- and/or PNA-array for analyzing diseases associated with the methylation state of genes, comprising at least one nucleic acid according to one of the preceding claims.
15. A method for determining genetic and/or epigenetic parameters for the characterisation, classification, differentiation, grading, staging, treatment and/or diagnosis of astrocytomas, or the predisposition to astrocytomas by analyzing cytosine methylations, characterized in that the following steps are carried out:
 - obtaining a biological sample containing genomic DNA
 - extracting the genomic DNA
 - in the genomic DNA sample, cytosine bases which are unmethylated at the 5-position are converted, by chemical treatment, to uracil or another base which is dissimilar to cytosine in terms of hybridization behavior;

- 29 -

- fragments of the chemically pretreated genomic DNA are amplified using sets of primer oligonucleotides according to Claim 8 or 9 and a polymerase, the amplificates carrying a detectable label;
16. A method for determining genetic and/or epigenetic parameters for the characterisation, classification, differentiation, grading, staging, treatment and/or diagnosis of astrocytomas, or the predisposition to astrocytomas by analyzing cytosine methylations, characterized in that the following steps are carried out:
- obtaining a biological sample containing genomic DNA
 - extracting the genomic DNA
 - in the genomic DNA sample, cytosine bases which are unmethylated at the 5-position are converted, by chemical treatment, to uracil or another base which is dissimilar to cytosine in terms of hybridization behavior;
 - fragments of the chemically pretreated genomic DNA are amplified using sets of primer oligonucleotides according to Claim 8 or 9 and a polymerase, the amplificates carrying a detectable label;
 - Identification of the methylation status of one or more cytosine positions
 - analysis of the methylation status of the cytosine positions by reference to one or more data sets.
17. The method as recited in Claims 15 or 16, characterized in that the amplification step preferentially amplifies DNA which is of particularly interest in astrocytoma or brain tissue, based on the specific genomic methylation status of brain tissues, as opposed to background DNA.
18. The method as recited in one of Claim 15 through 17, characterized in that the chemical treatment is carried out by means of a solution of a bisulfite, hydrogen sulfite or disulfite.
19. The method as recited in one of the Claims 15 through 18, characterized in that more than ten different fragments having a length of 100 - 2000 base pairs are amplified.

- 30 -

20. The method as recited in one of the Claims 15 through 19, characterized in that the amplification of several DNA segments is carried out in one reaction vessel.
21. The method as recited in one of the Claims 15 through 20, characterized in that the polymerase is a heat-resistant DNA polymerase.
22. The method as recited in Claim 21, characterized in that the amplification is carried out by means of the polymerase chain reaction (PCR).
23. The method as recited in one of the Claims 15 through 22, characterized in that the labels of the amplicates are fluorescence labels.
24. The method as recited in one of the Claims 15 through 22, characterized in that the labels of the amplicates are radionuclides.
25. The method as recited in one of the Claims 15 through 22, characterized in that the labels of the amplicates are detachable molecule fragments having a typical mass which are detected in a mass spectrometer.
26. The method as recited in one of the Claims 15 through 22, characterized in that the amplicates or fragments of the amplicates are detected in the mass spectrometer.
27. The method as recited in one of the Claims 25 and/or 26, characterized in that the produced fragments have a single positive or negative net charge for better detectability in the mass spectrometer.
28. The method as recited in one of the Claims 25 through 27, characterized in that detection is carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).
29. The method as recited in one of the Claims 15 through 28, characterized in that the genomic DNA is obtained from cells or cellular components which contain DNA, sources of DNA comprising, for example, cell lines, histological slides, biopsies, cerebrospinal

- 31 -

fluid, lymphatic fluid, tissue embedded in paraffin; for example, brain or lymphatic tissue and all possible combinations thereof.

30. A kit comprising a bisulfite (= disulfite, hydrogen sulfite) reagent as well as oligonucleotides and/or PNA-oligomers according to one of the Claims 2 through 4.
31. The use of a nucleic acid according to Claims 1 , of an oligonucleotide or PNA-oligomer according to one of the Claims 2 through 4, of a kit according to Claim 30, of an array according to one of the Claims 11 through 14, of a set of oligonucleotides according to one of claims 5 through 8 for the characterisation, classification, differentiation, grading, staging, treatment and/or diagnosis of astrocytomas, or the predisposition to astrocytomas.
32. The use of a nucleic acid according to Claims 1 , of an oligonucleotide or PNA-oligomer according to one of the Claims 2 through 4, of a kit according to Claim 30, of an array according to one of the Claims 11 through 14, of a set of oligonucleotides according to one of claims 5 through 8 for the therapy of astrocytomas.

Figure 1

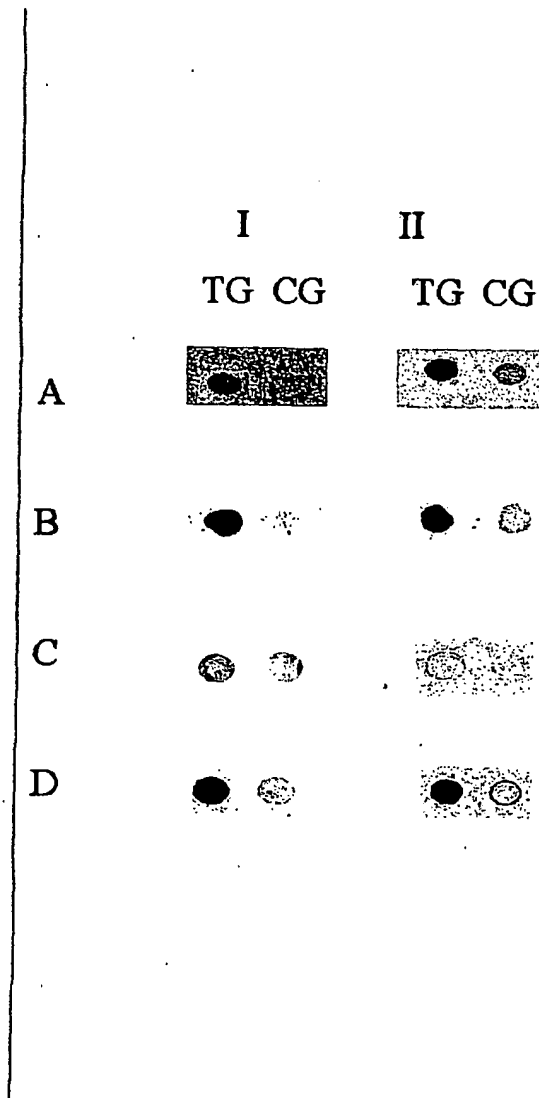


Figure 2A

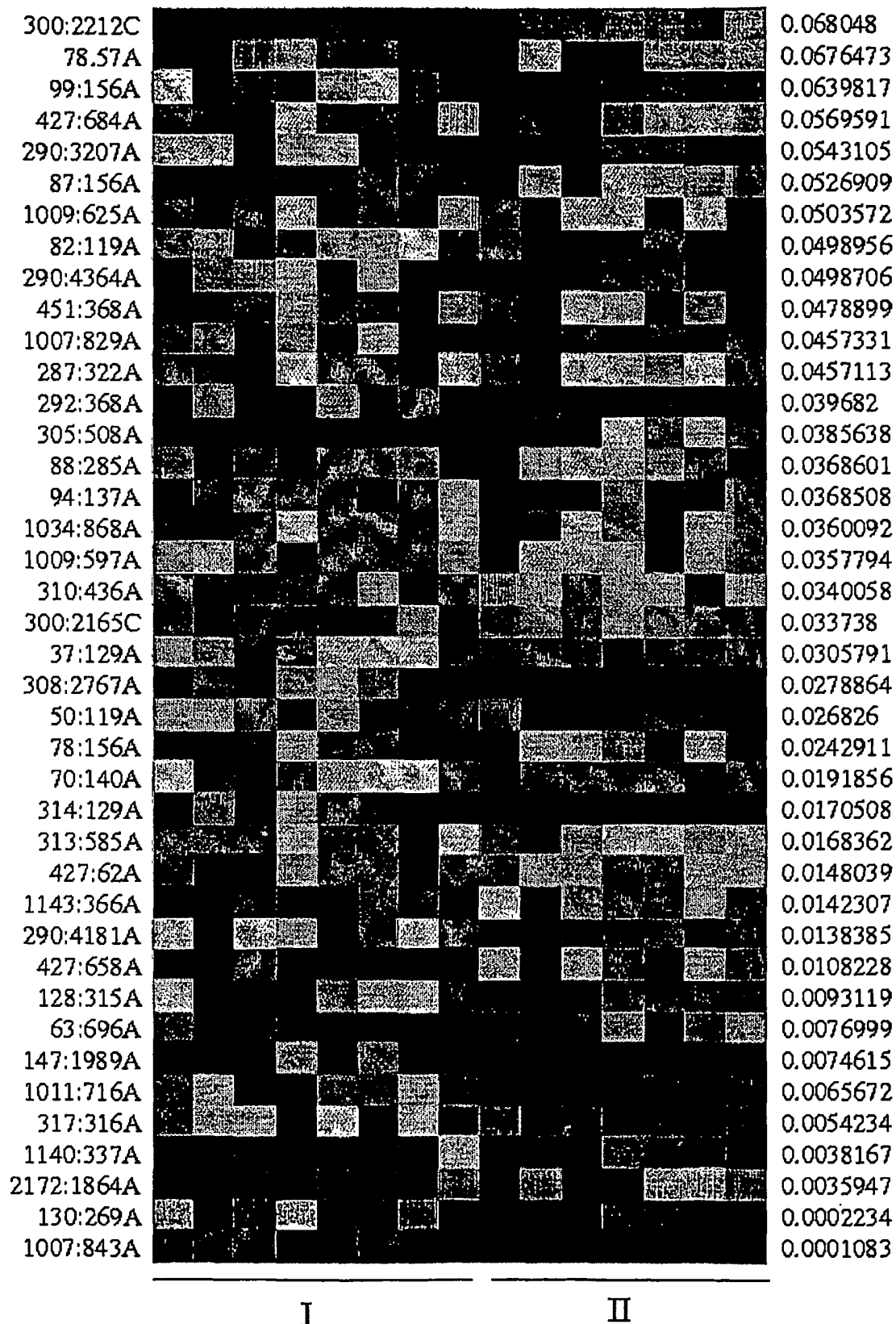


Figure 2A

3/5

Figure 2B

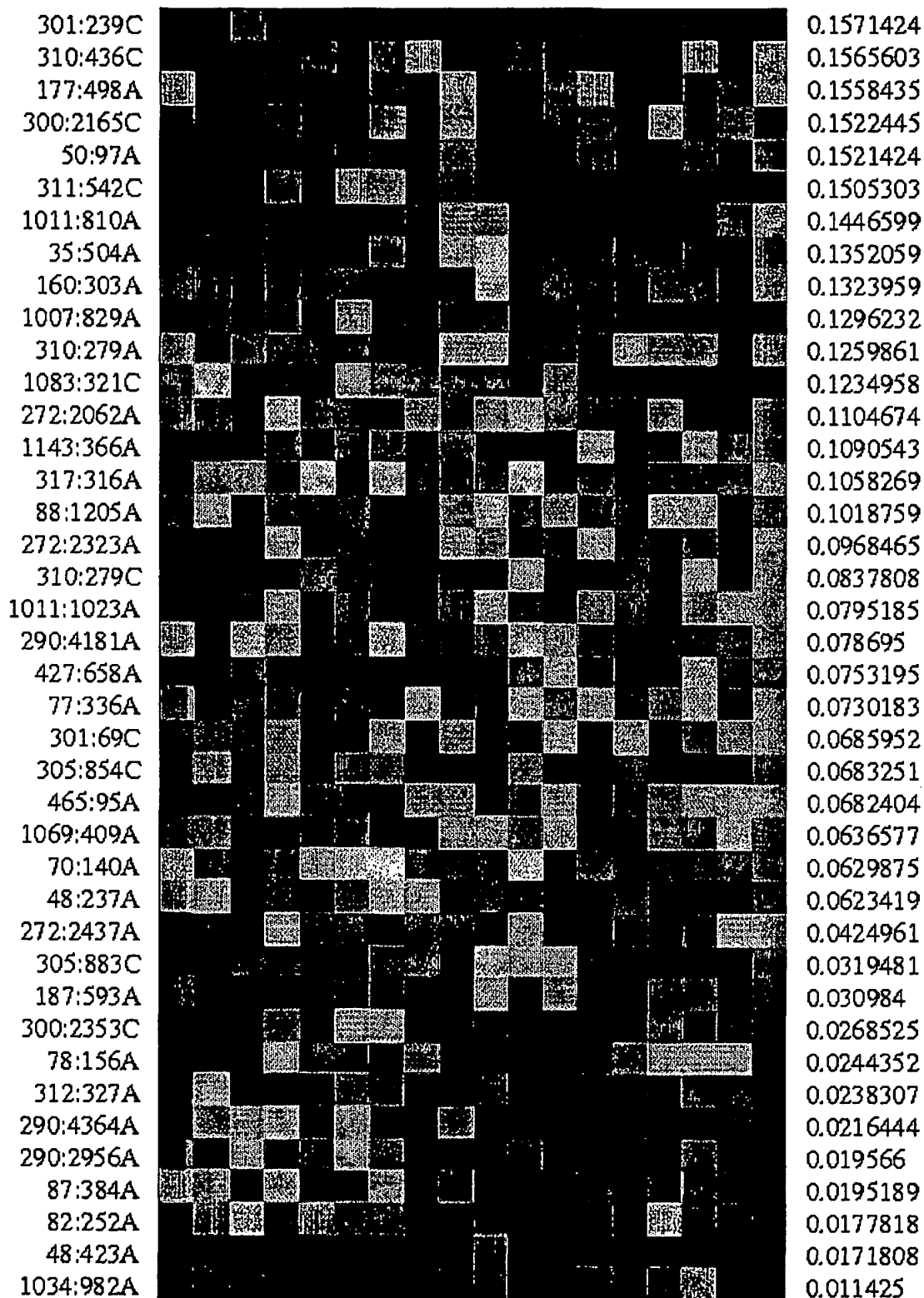


Figure 2B

I II

Figure 3

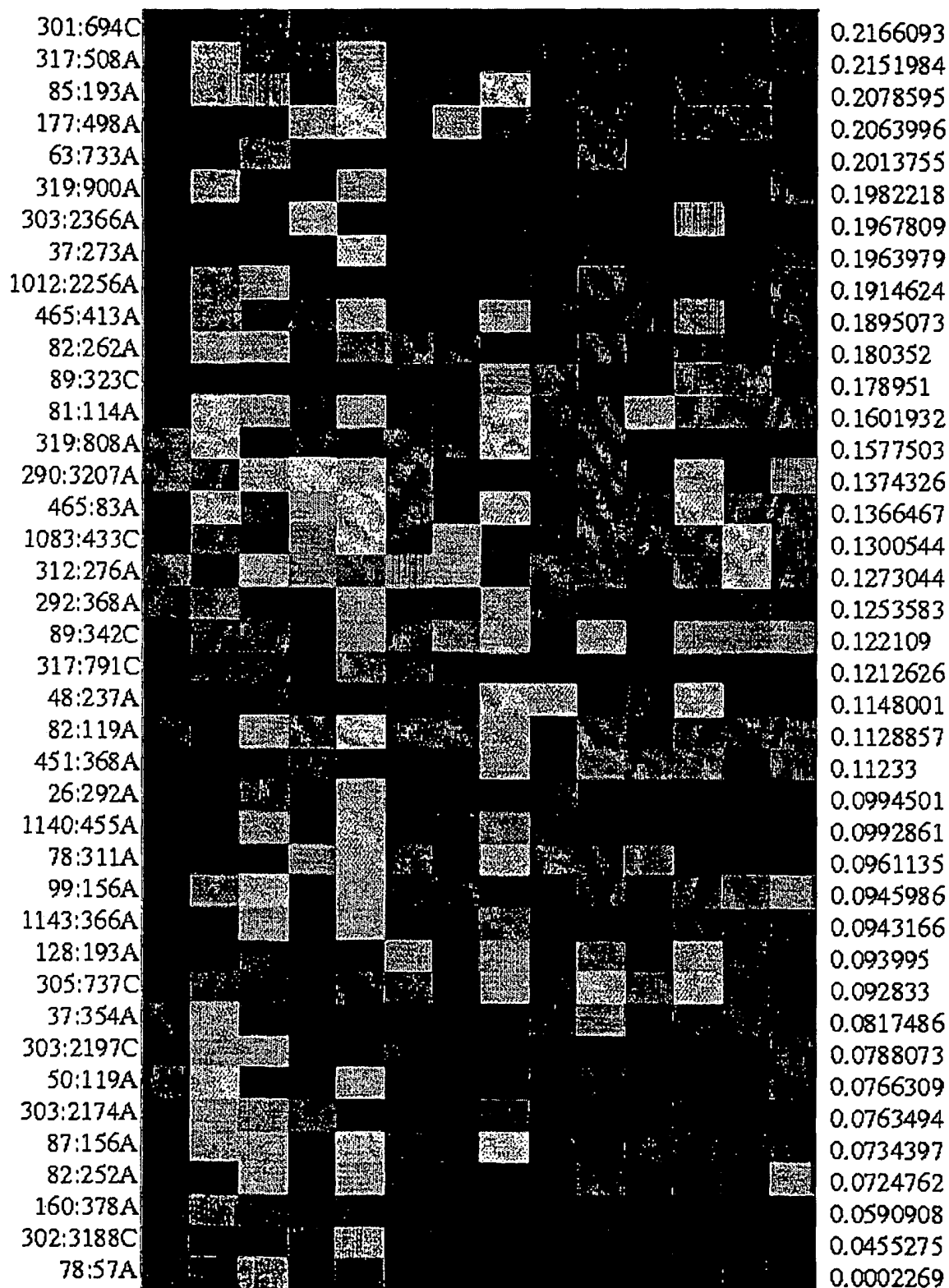


Figure 3

I

II

5/5

Figure 4

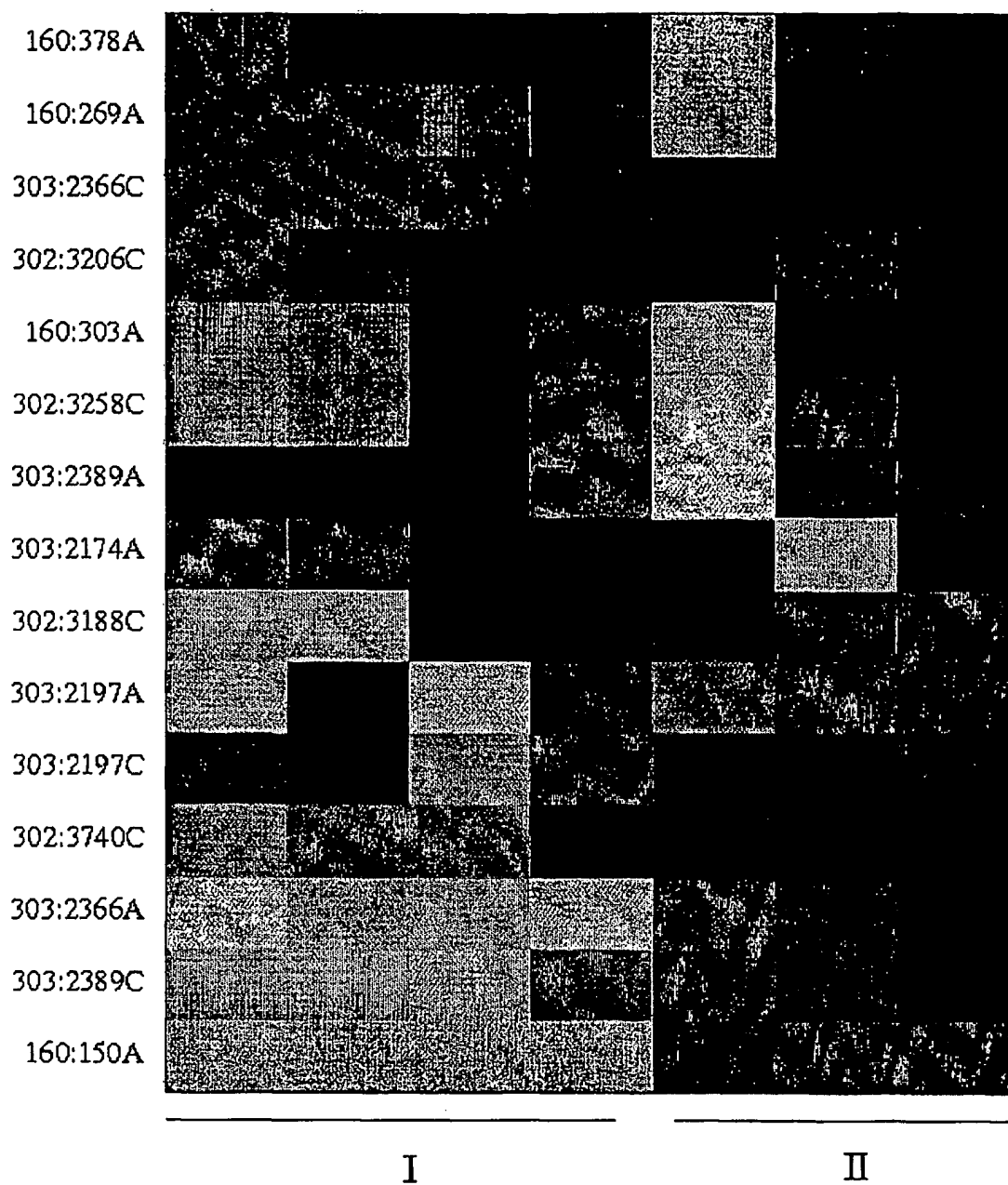


Figure 4

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(74) Agents: **SCHOHE, Stefan** et al.; Boehmert & Boehmert, Franz-Joseph-Strasse 38, 80801 München (DE).

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD AND NUCLEIC ACIDS FOR THE ANALYSIS OF ASTROCYTOMAS

(57) Abstract: The present invention relates to chemically modified genomic sequences, to oligonucleotides and/or PNA-oligomers for detecting the cytosine methylation state of genomic DNA, as well as to a method for ascertaining genetic and/or epigenetic parameters of genes for use in the characterisation, classification, differentiation, grading, staging, treatment and/or diagnosis of astrocytomas, or the predisposition to astrocytomas.

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INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/EP 01/07538

A. CLASSIFICATION OF SUBJECT MATTER		
IPC 7	C12Q1/68	B01J19/00 G01N33/483 C07K14/48
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 7 C12Q B01J G01N C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
WPI Data, EPO-Internal, SEQUENCE SEARCH, BIOSIS, MEDLINE, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	US 5 807 738 A (TAMAOKI TAIKI ET AL) 15 September 1998 (1998-09-15) SEQ ID 7 --- -/--	1-3
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
9 December 2002		14. 04. 03
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016		Authorized officer Luzzatto, E

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/07538

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X	<p>DATABASE EBI 'Online! Human AFP gene, intron with a partial ALU repeat, 20 March 1993 (1993-03-20) RUFFNER D.E. ET AL.: Database accession no. M37551 XP002224073 abstract & RUFFNER ET AL.: "Invasion of the human albumin-alpha-fetoprotein gene family by Alu, Kpn, and two novel repetitive DNA elements" MOLECULAR BIOLOGY AND EVOLUTION, vol. 4, 1987, pages 1-9,</p> <p>---</p>	1-3
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INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/EP 01/07538

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Inventor's Application No
PCT/EP 01/07538

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 01/07538

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the proscribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-32 (partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-32 (partially)

A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated DNA of a gene associated with astrocytoma according to sequence SEQ ID NO. 1 or 2, oligonucleotides having a sequence of at least 9 nucleotides identical or hybridizing to said DNA; a set of said oligonucleotides; the use of said set for detecting SNPs or the methylation state of cytosines in said nucleic acid; an array or set of said oligonucleotides fixed to a carrier; a method of producing said array; a method for diagnosis and/or therapy of astrocytomas or predisposition thereto using said oligonucleotides by analysing cytosine methylations; a kit comprising said oligonucleotides and bisulfite; and the use of said nucleic acid, oligonucleotides, set of oligonucleotides, array or kit for the diagnosis or therapy of astrocytomas.

2. Claims: 1-32 (partially)

A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated DNA of a gene associated with astrocytoma according to sequence SEQ ID NO. 3 or 4, oligonucleotides having a sequence of at least 9 nucleotides identical or hybridizing to said DNA; a set of said oligonucleotides; the use of said set for detecting SNPs or the methylation state of cytosines in said nucleic acid; an array or set of said oligonucleotides fixed to a carrier; a method of producing said array; a method for diagnosis and/or therapy of astrocytomas using said oligonucleotides by analysing cytosine methylations; a kit comprising said oligonucleotides and bisulfite; and the use of said nucleic acid, oligonucleotides, set of oligonucleotides, array or kit for the diagnosis of therapy of astrocytoma.

The same grouping applies to SEQ ID nos 5-120, giving inventions 3-60.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No.

PCT/EP 01/07538

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